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Title

What is the optimal treatment time for larval therapy? A study on incubation time and tissue debridement by bagged maggots of the greenbottle fly, *Lucilia sericata*

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Key Messages

- This investigation aimed to determine an optimal application time of bagged larval therapy treatments to maximise debridement by assessing larval growth and feeding over time.
- The mass of tissue digested by the larvae increased significantly in incremental 24 h incubation times up to 72 h, whilst larval mass increased only up to 48 h. Using larval growth and development to determine optimal application times could therefore result in an underutilisation of the debriding potential of larvae.
- An incubation time of 72 h was concluded to be the most effective use of bagged larvae at the test temperature of 32 °C. Shorter times appeared to underutilise the debridement capacity of the larvae and longer times offered little in terms of additional consumption.

Abstract

The effective use of larvae of the greenbottle fly, *Lucilia sericata*, in wound debridement requires a working knowledge of how feeding changes over time. Using a laboratory assay and bagged larval dressings, the effect of incubation time on larval feeding rates and body-mass was investigated for up to 120 h at 32 °C. The mass of tissue digested increased significantly in incremental 24 h periods up to 72 h, with no significant consumption occurring afterwards. Larval mass increased only up to 48 h. A further test comparing the efficacy of a single 96 h application of larvae against two consecutive 48 h applications found that the mass of tissue digested in the latter was 14.3% higher than the former, a difference that was statistically significant. Current clinical guidance suggests a four day application period for bagged larvae. Based on these results, an incubation time of 72 h (3 days) for bagged larvae would be most effective at the study temperature. However, it is acknowledged that wound temperature can vary, whereby feeding rates would likely differ. In view of this, we conclude that a period of 3-4 days is optimum for application of larvae, and current guidelines should be adhered to.

Key Words

Debridement, development time, larval therapy, *Lucilia sericata*, maggots

Introduction

Larval therapy is the therapeutic use of blowfly larvae to treat chronic, non-healing wounds. Delays in wound healing can be caused by a variety of factors including chronic disease, vascular insufficiencies, advanced age, neurological defects, nutritional deficiencies, or local factors such as infection, pressure, and oedema, and chronic wounds are characterised by a prolonged and self-perpetuating inflammatory response that can be difficult to manage (1,2). The failure to progress past this chronic state of inflammation can cause a cascade of abnormal tissue responses which can generate and amplify a hostile microenvironment inside the wound, resulting in the accumulation of cellular debris on the surface. Unrestrained proteolytic activity and a disturbed oxidant/antioxidant balance can also cause further damage to the surrounding tissue leading to infection and necrosis (3). To aid the process of wound healing and allow it to progress past the inflammatory stage, it is vital that this necrotic tissue is quickly and effectively debrided (4,5). In larval therapy, medicinal maggots are introduced into the chronic wound to undertake this process of debridement. Primarily, larvae of the greenbottle fly, *Lucilia sericata* (Diptera: Calliphoridae) are used.

The efficacy of larval therapy in debridement has been proved clinically. A number of clinical studies have been conducted to compare the efficacy of larval therapy to conventional treatment methods in debriding chronic wounds. A systematic review of the clinical studies of larval therapy noted twelve comparative studies, including six randomised controlled trials, from the years 2000–2014 (6). Based on the analysis of these twelve studies, the authors concluded that larval therapy is both more effective and more efficient in the debridement of chronic ulcers when compared with conventional treatments. They also associated larval therapy with other benefits, including quicker healing rate of chronic wounds, a shortened time to healing in ulcers, a longer antibiotic-free time period, decreased

amputation risk, and similar antibiotic usage compared with conventional therapies (6). A different meta-analysis of seven clinical studies from 1995–2009, including three randomised controlled trials and four non-randomised trials suggested that whilst larvae were effective in debridement, there was not enough evidence to show that they were more effective than conventional treatments (7).

In addition to debridement, the therapy is also associated with numerous secondary benefits. The larvae of *L. sericata* have been shown to possess significant antibacterial capabilities, not only by the removal of necrotic tissue, but also through the antimicrobial action of their secretions (8–10). Larval therapy has also been implicated in promoting the growth of and appearance of extracellular matrix or granulation tissue which may help to enhance new tissue formation (11,12). In addition, other noted benefits of larval therapy have been suggested including reperfusion, reduction in inflammation, and antifungal properties (13,14).

The ability of medicinal maggots to debride is primarily attributed to the way in which they feed. Being necrophagous by nature, the larvae break down and consume necrotic tissue enzymatically by a process of extracorporeal digestion. This is achieved by the release of excretions/secretions containing a mixture of proteolytic, glycolytic, lipolytic, and nuclease enzymes onto the tissue surface, causing liquefaction and digestion of the necrotic tissue which is subsequently ingested (15–19). However, as effective as this feeding mechanism may be, the larvae cannot feed indefinitely. Newly hatched larvae will feed through three larval instar stages, before reaching a pre-pupal wandering stage (20). This wandering stage is characterised by the cessation of feeding and migration away from the feeding site as the larva begins to search for a suitable location to begin pupation (21). Due to the ability of the

larvae to digest tissue being confined to these stages of development, a single treatment of larval therapy will only be effective in debridement for this limited timespan.

The current investigation focussed on the use of bagged larvae. In this method, the larvae are sealed inside a porous polymer bag which allows for the flow of larval excretions and secretions and the liquefied necrotic tissue and wound exudate out of and into the bag whilst keeping the larvae contained (22,23). This facilitates an ease of use in the application and removal of the larvae (24).

Recommended application times for bagged larvae are variable. Guidance from medicinal larvae producer BioMonde (Bridgend, U.K.) recommends an application period of a maximum of four days for their bagged larvae products (25), though Thomas (21) suggests that treatment times using bagged larvae can last 2–5 days. Using a wound model, Blake *et al.* (26) found that significantly more tissue was metabolised after four days than after three, and therefore recommended a four-day application period. More recently, Čičková *et al.* (27) found that larval growth ceased after 48 h when using a similar model and recommended an application period of 48–72 h for bagged larvae (24,27). Controlled trials using bagged larvae have also used application times of 2–3 days (24,28) and 3–4 days (29). Some research has also previously been undertaken in the area of larval development duration, though much of it relates to their usefulness as a forensic tool. These studies tend to focus on minimum development times and give little information about how the larvae feed over time (30–33), so attempting to extrapolate useful larval therapy application times from these is only of limited value.

Debridement is often an imperative of wound treatment and any delay in the time to debridement can, in turn, delay the wound healing process (34). A working knowledge of the feeding activity of medicinal larvae over time is, therefore, essential for the effective application of this treatment. Considering the significance of debridement to the wound treatment method and a current lack of consensus regarding a recommended application time of bagged larval therapy products, a clearer understanding of feeding and digestion processes of medicinal larvae over time is needed.

The aims of this study were, first, to investigate the activity of bagged medicinal grade *L. sericata* larvae over the course of an application period of up to 120 h in a laboratory assay and, second, to determine the efficacy of larval feeding processes over this time. Additionally, we sought to investigate whether two treatments of bagged larvae applied for 48 h, one being replaced by the other, would result in greater total consumption than a single treatment applied for 96 h. Such information would prove significant in understanding the digestive processes of larvae over time and in formulating an optimal application period of this larval therapy treatment.

Methods

This study made use of a 'larval activity assay' as described previously (35). The assay uses an aerated container fitted with sponge, containing a mass of minced pork loin upon which the larvae feed. Efficacy in digestion is then indicated by the mass of tissue consumed by the larvae over time. Medicinal grade larvae were supplied by BioMonde (Bridgend, U.K.) in vials, each containing approximately 200 individuals, along with bagged larval dressings, measuring 25 × 40 mm, and foam spacers, measuring 8 × 8 × 10 mm. Upon set-up of the tests, the larvae were second instar.

For every test iteration, fifty larvae were counted into each BioBag50, along with a foam spacer, before being heat sealed. The assay was constructed as described previously (35), but with some modifications. A mass of 15 g of minced pork tissue was used as the feeding substrate in this instance as this was found to be enough to ensure that there was feeding material available throughout the 120 h. The construction of the assay was also modified to include an absorbent cotton pad placed over the BioBag, which acted to absorb excess fluid produced by the larval action, reducing the risk of larvae suffocating inside the dressing.

Time series test

The assays were incubated at 32 ± 1 °C for five time periods: 24, 48, 72, 96, and 120 h with twelve repeats conducted for each experiment, along with three control repeats that contained no larvae. This temperature was used as previous research has indicated this to be the approximate average wound surface temperature (26,36,37). After the designated time period, the assay container was removed and the BioBag opened. The larvae were counted out of the bag, the total mass of larvae was recorded, and the mean mass per larva calculated. The dry

mass of pork tissue remaining was also recorded, which was obtained by placing it in a drying incubator at 70 ± 2 °C for 48 h. Dry mass was recorded to remove inconsistencies in tissue mass caused by water loss over the five day test period. An estimated initial dry mass was obtained by drying separate 15 g portions of minced pork. Five samples were dried from each batch of pork loin used and the mean dry mass calculated from these samples were used as the estimated initial dry mass.

Comparison test

A comparison test was also conducted to assess the feeding efficacies of two separate treatments over 96 h. In one treatment, a single BioBag was used for the duration of the 96 h test, whilst in the other, the BioBag was replaced with a fresh bag containing a fresh batch of larvae after the first 48 h of feeding. Results were then collected at the end of the 96 h, recording the same parameters as those in the time series test. Larval mass data was also recorded for both instances of the 48 h treatment. A total of 9 repeats were conducted for each treatment. The process of replacing the BioBags was carried out by removing the assay containers from the incubator after 48 h, opening the lid and then removing the bag with thumb forceps. The new BioBag was then placed on top of the pork tissue with the same orientation as the previous bag, before being resealed and placed back in the incubator. In tests where the same bag was used for the duration of the 96 h, these actions were mimicked, but with the same bag being removed and then replaced.

Statistical analysis

Data analysis in the time series test consisted of separate analysis of variance (ANOVA) tests to determine an overall effect of incubation time on both the mass of tissue lost and the mean mass per larva. To determine more specifically the effect of each successive 24 h increment,

post-hoc Tukey multiple comparison tests were subsequently performed to compare the mean of each increment with those of every other. Significant differences between means were indicated at the 0.05 level of significance. These were conducted for both the mean mass of tissue lost and the mean mass per larva. For the comparison test, data were analysed using unpaired t-tests. These were conducted to compare the mean masses of tissue lost in the two treatments. Separate t-tests were also conducted comparing the mean mass per larva of both the first 48 h application of larvae with the mass per larvae from the 96 h application of larvae, and also for the second 48 h application of larvae against the 96 h application. One final t-test was conducted comparing the mean mass per larvae of both 48 h applications.

Results

Time series test

Overall, incubation with larvae over 120 h was found to have a significant impact on the mass of tissue lost, $F(5, 66) = 331.8$, $p < 0.001$. The mass of tissue removed over time increased steadily over the first 72 h (Figure 1A). The multiple comparison tests revealed that the differences between these time periods were statistically significant at the 0.05 level of significance. After 72 h the increase in the mass of tissue loss was much less pronounced, and at 120 h, tissue loss did not change at all (Figure 1A). Of the total mass of tissue removed over the five day period, 92.1 % was removed in the first 72 h. No significant differences were observed after the 72 h time point (i.e. the masses of tissue lost at the 72, 96, and 120 h time periods were not significantly different from each other). Control tests containing no larvae saw a relatively small loss of mass over time with (mean \pm SEM) 0.31 ± 0.06 g lost after 24 h, and rising in 24h increments to 0.57 ± 0.05 g after 120h.

The trend in mean mass per larva saw an increase over time up to 72 h and then gradually decreasing afterwards (Figure 1B). Incubation time over the whole 120 h test period was found to have a significant impact on the mass per larva, $F(5, 66) = 228.4$, $p < 0.001$. Mass per larva increased steadily over the first 48 h, with the multiple comparison tests showing significant differences between the mean masses at 0, 24, and 48 h. Mean larval mass increased again between 48 and 72 h, but to a lesser degree, and the difference was not statistically significant. Mean mass then decreased after the 72 h point. Although the difference from the 72 h time point was not significantly lower at 96 h, the decrease was statistically significant at 120 h.

Comparison test

A total (mean \pm SEM) 2.44 ± 0.05 g dry mass of tissue lost in the 96 h treatment, compared with 2.79 ± 0.07 g of tissue was lost in the two successive 48 h treatments, showing an increase of 14.34% (Figure 2A). Using an unpaired t-test, the difference was found to be statistically significant, $t(16) = 4.097$, $p < 0.001$. The mean mass per larva was 40.38 ± 0.53 mg after the 96 h treatment. In the separate successive 48 h treatments, the mean larval mass was 37.59 ± 1.92 mg after the first 48 h application and the replacement larvae were 38.44 ± 2.23 mg after the second 48 h application (Figure 2B). The mean larval mass of the 96 h treatment was not significantly different from either the first 48 h treatment, $t(16) = 1.401$, $p = 0.180$; or the second 48 h treatment, $t(16) = 0.842$, $p = 0.412$. There was also no statistically significant difference between the mean larval masses of the two 48 h applications, $t(16) = 0.291$, $p = 0.775$.

Discussion

Mass of tissue lost

Debridement is the primary function of larval therapy and it relies on the ability of the larvae to consume dead tissue. Maximising the efficacy of larval therapy treatments, therefore, requires a good knowledge of the length of time for which the larvae are active. In the present study, larval activity was determined, firstly, by the mass of tissue consumed over time. The results of this study indicate an active feeding period of up to 72 h at the study temperature of 32 °C. The primary explanation for the change in consumption rates over time is due to the developmental processes of the larvae. The active feeding period of larval development has been described as a “race against time” where, in response to the limited availability of food, larvae must obtain resources as quickly possible to gain the critical weight required to successfully complete metamorphosis (38–40). It is likely for this reason that the rates of consumption are highest in the earlier incubation period. The subsequent slowing in the rate of tissue removal indicates the point at which the larvae cease feeding and begin their wandering behaviour, a process that is largely dictated by temperature (31,33,41). In the present study it appears the cessation of feeding occurred in most individuals between 48–72 h as the subsequent 24 h period showed no significant increase in tissue removal. Control tests containing no larvae saw a relatively small mass of tissue loss over time possibly due to bacterial and autolytic degradation.

Larval development has been investigated in a number of ways in past studies. A majority looked either at the time taken to develop certain characteristics or at larval size/mass over time (31,33,41,42). Few, however, have considered consumption rates, with only one previous study noted, which found that significantly more tissue was digested after four days than after three (26). Those results differ somewhat to these in the present study. Although in

the present study the mean dry mass of tissue lost after 96 h was greater than at 72, the rate of loss was far lower than that seen in previous 24 h intervals and the difference in tissue loss between 72 and 96 h was found not to be statistically significant. Differences between the results may be due to wet mass measurements being recorded by Blake *et al.* (26) which may have caused inconsistencies in the measurements due to desiccation of the feeding material over time – a limitation which has been suggested previously (27). The impact of water loss was addressed in the present study by the use of dry mass measurements. In this case, there appeared to be little benefit of incubating the larvae for 96 h rather than 72 in terms of tissue digestion.

Mass per larva

Larval mass is closely linked to activity as any changes will be correlated with food intake. In the present study, larval mass increased significantly up to 48 h, but showed no significant growth afterwards, and began to decrease after 72 h. The lack of growth after 48 h was also observed in the comparison test where there was no significant difference observed in the mass per larva between the larvae that were incubated for 96 h and either of those incubated for the 48 h periods. These results are also supported by those from a previous study, which found that larval growth ceased after 40–48 h when incubated in simulated wound conditions (27).

In the present study it was found that larval growth did not always coincide with feeding. Larvae were found to still be effective in consuming tissue up to 24 h after they had stopped showing any significant increase in mass. After the significant increases in growth up to 48 h, it seems that rates of feeding in the following 24 h were enough only to maintain body mass. Increases in body mass may also have been offset by emptying of the larval crop as they

began to enter the wandering stage of development. Using only growth data to determine larval therapy application times, therefore, could lead to underutilising the larvae as this would result in their being removed at a time where they are still feeding and capable of debriding tissue. This is significant as previous studies have used larval growth as a parameter to justify recommendations of larval therapy application times. For example, Čičková *et al.* (27) noted that larval growth ceased after 40–48 h and went on to recommend this time period as that which was most appropriate for larval therapy applications using the free-range larvae method. The recommendation given for bagged larvae was longer, at 48–72 h, but only as it was noted in a previous study that bagged larvae appeared to grow more slowly in certain types of wounds (24). As debridement is a key aim of larval therapy it is important to consider consumption data also when considering optimal application times.

Based on the results of this investigation, the application of bagged larvae for 48 h would result in an underutilisation of the larvae as they are capable of consuming significantly more tissue for a further 24 h. This is also somewhat exemplified by data from the comparison test. In this test, the two consecutive 48 h treatments resulted in an increase in the mass of tissue removed over a single 96 h application of 14.3%, which was statistically significant. From a statistical point of view, one can conclude that the use of consecutive 48 h applications was more efficacious than a single 96 h application as it resulted in a greater mass of tissue removal. From a practical perspective, however, one could question whether the relatively modest increase in tissue removal justifies the costs that would be associated with applying two rounds of the treatment as opposed to one. Considering the consumption data both here and in the full 120 h test, it seems that a 72 h application would be a more effective use of the larvae at this temperature.

Impact of temperature

Temperature is a primary factor determining larval development rates with higher temperatures resulting in faster development times regardless of food availability (30,31,33,40,41). The temperature that the larvae experience determines for how long they will be actively feeding. Therefore, in a larval therapy context, wound temperature plays a key role in determining for how long the debridement process will likely be effective.

A temperature of 32 °C was chosen in this study as previous research has indicated this to be around the average wound surface temperature (26,36,37). However, it is acknowledged that the wound bed can exhibit a range of temperatures that can vary depending on body location, coverings or dressings used, and levels of inflammation or vascularity, with one study measuring wound surface temperature of 266 wounds finding a range of 25.3–37.3 °C (37).

Whilst this investigation gives an insight into the duration of larval feeding at average wound temperatures, further investigation into the effect of the range of temperatures that can be exhibited by chronic wounds on larval consumption rates would also be useful for better determining the optimal application times of larval therapy treatments. These differences in temperature could significantly impact for how long the larvae will be effective.

Although the present investigation found an active feeding period of up to 72 h at the study temperature of 32 °C, the current clinical recommendation of up to 4 days (25) can still be considered sensible, since wounds that present a lower temperatures may cause slower larval development and a longer period of active feeding.

Future studies

Efforts were made for the assay to mimic aspects of a chronic wound so that results were as representative as possible to the clinical situation. However, the assay is not a substitute for clinical trials and the limitations of this in-vitro study should be acknowledged. There is no guarantee of a direct link between results found using this assay and those in the clinical situation, and although the findings of this study can aid in influencing future protocols for clinical application, further clinical investigation would be necessary to confirm optimal application times of medicinal larvae. Additional work may also look to consider secondary benefits of larval therapy, such as antimicrobial action, in-vitro by the inoculation of the meat substrate with pathogenic bacteria and then examining the performance of the larvae in the presence of these bacteria.

This study investigated only the use of bagged larvae. Recommended application times vary depending on the larval therapy product, with loose larvae products generally being recommended shorter application times than bagged larvae (21,27). A similar future study considering the optimal application times of loose larvae would, therefore, also be useful.

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Figures

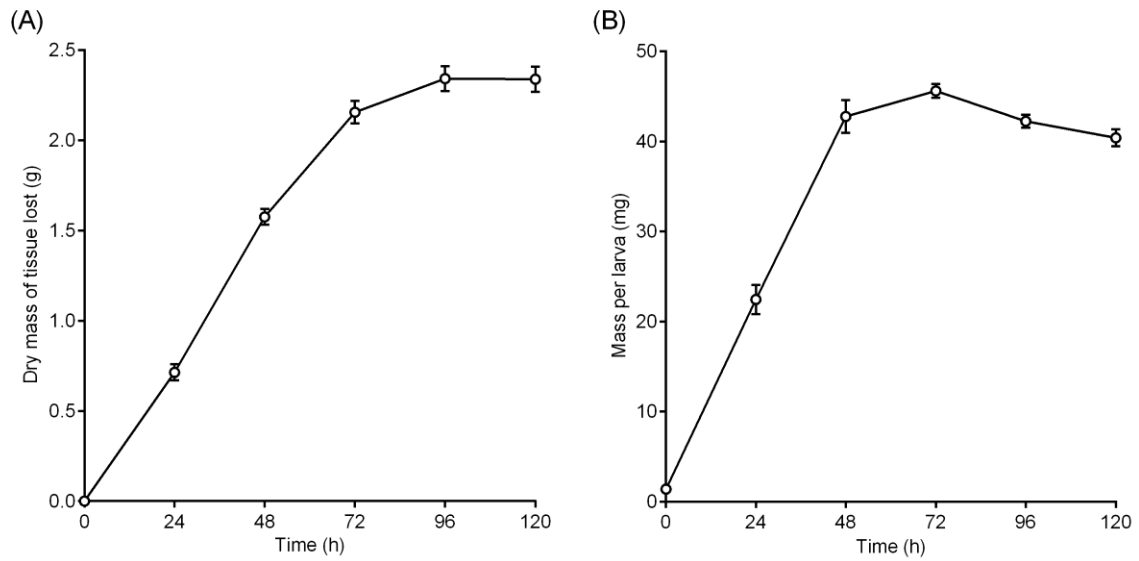


Figure 1. Mean \pm SEM (A) total dry mass of pork tissue lost, and (B) mass per larva from artificial assay tests incubated for different time periods at 32 ± 1 °C. Test experiments using BioBags containing 5 larvae/cm² (n = 12 per time period) are shown in circles, and control experiments using BioBags containing no larvae (n = 3 per time period) are shown as triangles.

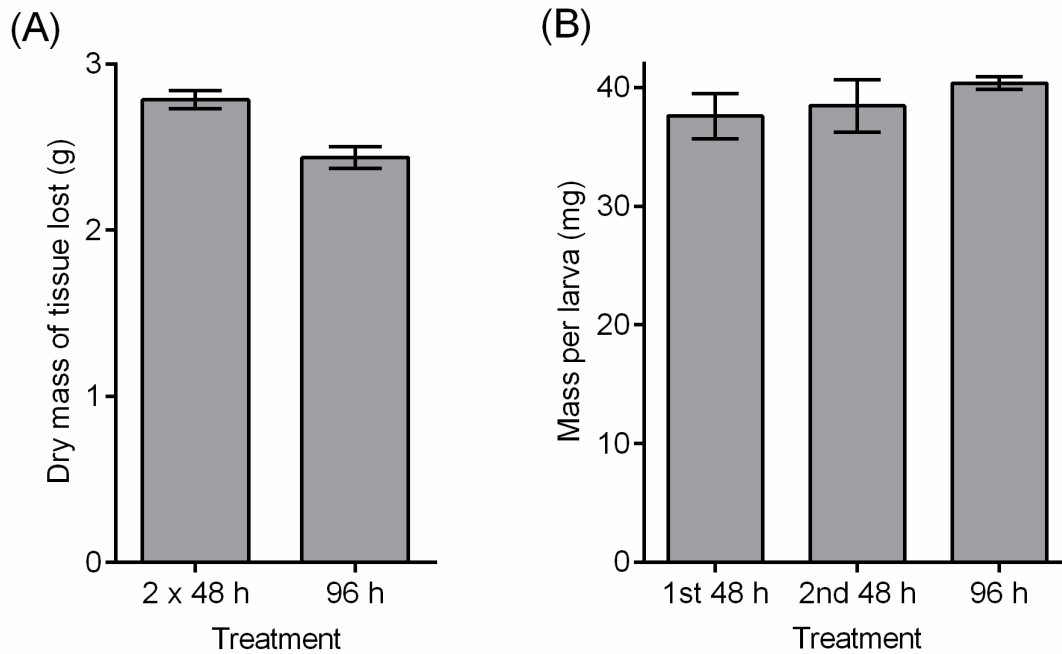


Figure 2. (A) Mean \pm SEM dry mass of pork tissue lost in artificial assay tests after 96 h feeding, comparing a treatment where the BioBag was replaced with a fresh bag after 48 h ('2 \times 48 h' treatment) and a treatment where the same BioBag was used for the full 96 h duration ('96 h' treatment). (B) Mean \pm SEM mass per larva from assay tests after different periods of feeding, including larvae from the initially applied bag in the '2 \times 48 h' treatment ('1st 48 h'), larvae from the bag applied second in the '2 \times 48 h' treatment ('2nd 48 h'), and larvae from the from the bag applied in the '96 h' treatment. All tests were incubated at 32 ± 1 °C with BioBags containing 5.0 larvae/cm². n = 9 per treatment.